Scalable screening assay for compounds that enhance cardiomyogenesis of ESC derived cardiac progenitor cells

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Tiivistelmä – Referat – Abstract

During and after myocardial infarction, millions to a billion cells die off. Scar tissue formed by fibroblasts replaces the injured myocardium during recovery. While the newly formed tissue is durable and prevents rupture of the heart, it doesn´t contribute to pump function. Depending on the extent of cardiomyocyte loss, the remaining functional myocardium get strained. Adult mammalian heart has inadequate capacity to regenerate after such injury. In case of sustained substantial increase in workload, the compensatory mechanisms turn into pathological processes including excessive fibrosis and myocyte apoptosis. The progressive decline of hearts contractile function results in heart failure (HF).

Current drug treatments for managing HF aim to prevent progression of the disease and relieve symptoms. ACE inhibitors, beta blockers and diuretics are effective along with healthy lifestyle. No practical treatments are available to restore cardiac function yet.

Human myocardium normally regenerates, but only 1% or less of myocytes get replaced yearly. Heart's resident stem/progenitor cells (CPCs) likely play a role in the turnover. The aim of this study was to develop a screening method to identify small molecules that possibly promote differentiation of cardiac progenitor cells to cardiomyocytes. Cell population differentiated from mouse embryonic stem cells (mESCs) was used as a model for CPCs. Directed differentiation protocol of mESCs used here promotes commitment to cells of cardiac mesoderm, part of which will further differentiate to cardiac progenitors. The resulting population at day 6 is heterogenous but many of these cells are progenitors that turn into cardiomyocytes (CMs) by day 8.

10 000 cells per well are plated on 384 well plates at day 5. Test compounds are added at day 6 and removed day 8 for effect in progenitors and day 7-9 for effect in early cardiomyocytes. 0,1% DMSO is used as vehicle and Wnt pathway inhibitor XAV939 as positive control. The effects are quantified with plate reader on day 9.

E14 derived mESC reporter line was used. Myl2v-eGFP + SMyHC3-RFP double reporter line allows the specific identification of ventricular CMs with green fluorescence and atrial CMs with red fluorescence. Plate reader measures the total fluorescence of the wells at 485/520nm on day 9, which is used as a readout for ventricular CMs. The fluorescence intensity depends on the amount of GFP+ cells but also on the level of Myl2v expression. Atrial CMs could be quantified similarly but the population doesn´t contain enough RFP+ cells.

The assay was shown to reliably point out 'hits' that have a strong effect. Any compounds that only produce a moderate effect could be a false negative, however. The effect on cardiac progenitors could likely be increased by simply adding the compounds earlier on day 5. Variability of key reagents causes the main technical troubles through unpredictably affecting cytokine concentrations which decreases the amount of cardiac progenitors. Partially similar screening assays are being used by the big pharma where they cryopreserve progenitors in bulk for later use, thus simplifying and speeding up their method. Same approach could be adopted.

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Skaalautuva seulontamenetelmä yhdisteille, jotka tehostavat sydämen kantasolujen kehittymistä sydänlihassoluiksi

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Tiivistelmä – Referat – Abstract
Sydäninfarktin aikana ja sen seurauksena miljoonista miljardiin solua kuolee. Fibroblastien muodostama arpikudos korjaa vaurioituneen sydänlihaskudoksen. Vaikka muodostunut arpikudos on kestävä ja estää sydämen repeämisen, se ei osallistu veren pumppaamiseen. Kuolleiden sydänlihassolujen määrästä riippuen terve lihaskudos kompensoi tekemällä kovemmin töitä, ja rasittuu. Aikuisen nisäkkään sydän ei kykene palautumaan kudostuhosta. Jos sydänlihaskudoksen työmäärä kasvaa merkittävästi, kompensatoriset mekanismit johtavat lopulta tilanteeseen jossa terve sydänlihaskudos menettää toimintakykyään. Sydämen toiminnan heikentyessä etenevästi syntyy sydämen vajaatoiminta.

Nykyiset sydämen vaajaatoiminnan lääkehoidot tähtäävät oireiden helpottamiseen ja estämään tautiprosessin etenemisen. ACE:n estäjät, beetasalpaajat ja diureetit ovat tehokkaita yhdistettynä terveellisiin elämäntapoihin. Käytännöllisiä keinoja sydämen toiminnan korjaamiseksi ei kuitenkaan vielä ole saatavilla.

Ihmisen sydämen lihassolut uusiutuvat alle 1 prosentin vuosivauhtia. Sydämen kanta- tai esiastesolut (CPC) ilmeisesti osallistuvat tähän vanhojen lihassolujen korvautumiseen uusilla. Tämän tutkimuksen tavoitteena oli kehittää seulontamenetelmä tunnistamaan pienmolekyylejä, jotka mahdollisesti lisäävät sydämen esiastesolujen erilaistumista sydänlihassoluiksi. Testattavana solupopulaationa käytettiin hiiren alkion kantasoluista erilaistettuja esiastesoluja. Käytetty erilaistamisprotokolla ohjaa kantasolut kehittymään sydänmesodermin soluiksi, joista osa erilaistuu edelleen sydämen esiastesoluiksi. Tuloksena saadaan heterogeeninen solupopulaatio, josta suuri osa edustaa sellaisia esiastesoluja, jotka voivat muodostaa sydänlihassoluja.

10 000 solua per kuoppa siirretään 384-kuoppalevylle. Testattavat yhdisteet lisätään 24 tai 48 tunnin kuluttua siirtohetkestä ja altistusta jatketaan kahden päivän ajan.

Tutkimuksessa käytetty kahden reportterigeenin kantasolulinja Myl2v-eGFP + SMyHC3-RFP mahdollistaa kammion kardiomyosyyttien tunnistamisen niiden vihreästä markkerista, sekä eteisen kardiomyosyyttien vastaavasti punaisesta markkerista. Koeyhdisteille altistuksen jälkeen mitataan levylukijalla kunkin kaivon kokonaisfluoresenssi aallonpituudella 485/520nm ja tätä lukemaa käytetään osoittamaan kammiokardiomyosyyttien määrää. Fluoresenssin intensiteetti rippuu GFP-positiivisten solujen määrästä, mutta myös Myl2v-geenin ekspression voimakkuudesta. Eteisen kardiomyosyyttejä voitaisiin mitata vastaavasti, jos mitattava populaatio sisältäisi tarpeeksi suuren määrän RFP+ -soluja.

Kehitetyn menetelmän osoitettiin luotettavasti tunnistavan molekyylejä, joilla on vahva vaikutus. Kohtalaisen vaikutuksen aikaansaavat molekyylit voivat kuitenkin tuottaa väärän negatiivisen tuloksen testissä. Esiastesoluihin kohdistuvan vaikutuksen suuruutta voidaan todennäköisesti lisätä yksinkertaisesti ainakin aloittamalla käsittely hieman aiemmin. Menetelmän suurimmat tekniset ongelmat johtuvat keskeisten reagenssien laatuvaihteluista, jotka vähentävät esiastesolujen määrää alustoilla. Samankaltaisia seulontamenetelmiä on lääketeollisuudenkin käytössä, mutta yleisesti menetelmiä yksinkertaisettu käyttämällä kryologisesti säilytettyjä esiastesoluja.

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Abbreviations

Mesp1	Mesoderm posterior 1
BMP	Bone Morphogenetic Protein
CPCs	Cardiac progenitor cells, including what is often referred to as cardiac stem cells elsewhere
DMSO	Dimethyl Sulfoxide
E6.5	Embryonic age after fertilization
EB	Embryoid Body
eGFP	Enhanced Green Fluorescent Protein
FGF	Fibroblast growth factor
FHF	First heart field
Myl2	Myosin regulatory light chain 2, ventricular isoform
mESCs	mouse embryonic stem cells
PS	Primitive streak
SHF	Second heart field
Shh	Sonic hedgehog
TF	Transcription factor

1 Introduction

Cardiovascular diseases and heart failure among them account for significant mortality. Adult mammalian heart has inadequate capacity to regenerate after large injury such as myocardial infarction. During and after myocardial infarction, million to a billion cells may die off. Scar tissue formed by fibroblasts replaces the injured myocardium during recovery. While the newly formed tissue is durable and prevents rupture of the heart, it doesn´t contribute to pump function. Depending on the extent of cardiomyocyte loss, the remaining functional myocardium gets strained. In case of sustained substantial increase in workload, the compensatory mechanisms turn into pathological processes including excessive fibrosis and myocyte apoptosis. The progressive decline of hearts contractile function results in heart failure (HF).

Chronic heart failure progresses trough variety of mechanisms, but the core process is the death of more cardiomyocytes. With the advent of new treatments, disease progression can be slowed down or even stopped. However, the shortage of functional myocardium remains an underlying problem that needs to be addressed. Several ways to restore cardiac function have been proposed. First approach includes activating endogenous processes to enhance production of new cardiomyocytes (CMs) from either old CMs or heart's endogenous cardiac progenitor cells (CPCs). Alternatively cells from numerous sources can be transplanted into myocardium. Reprogramming of other cell types to generate new CMs represent yet another option.

Ways to drive innate repair processes in heart involve modulating relevant signal pathways. RNA therapeutics and peptide drugs can be utilized in addition to small molecules. Regenerative approaches based on pharmacological means are attractive.

Basic research provides insight into mechanisms that could aid in deciding effective targets for regenerative medications. In particular, heart's embryonic development and robust reparative capacity of other vertebrate species have instructed mechanisms,

which could play a role in human heart repair. Heart's endogenous progenitor cells (CPCs) are one of the most important targets in developing new kinds of therapies.

Development of novel therapeutics for cardiac repair is constrained by lack of effective research tools available. Therefore, this work seeks to establish a method to search for novel compounds that could affect the pool of CPCs endogenous to adult heart tissue. Phenotypic screening approach serves to indiscriminately identify putative compounds driving generation of CMs regardless of their target. Because the signals are not all that well known, this shotgun approach could yield useful hits.

2 Basic research may instruct development of novel therapeutic modalities

The therapies available today can effectively reduce hearts pathological remodeling and progression of chronic heart failure. ACE inhibitors, aldosterone antagonists, vasodilators and beta blockers are the prevalent medications used to increase life expectancy of the patients (Szema et al. 2015). Recent progress in intervention techniques of acute myocardial infarction (MI) has significantly improved patient survival. Challenge is to find treatments to restore cardiac function by reversing myocardial damage.

Cardiomyocytes in the heart do in fact regenerate, just very slowly. Stem or progenitor cells reside in the heart and at least some of them have been shown to be able to proliferate into cardiomyocytes among other cell types. This is a promising possibility. The signals that direct hearts regenerative processes are convoluted, however.

It seems that in absence of injury a portion of differentiated mononucleated cardiomyocytes are proliferating and not the CPCs. CPCs probably play a role in this process though. In adult mammals, CMs don´t proliferate in response to myocardial injury nor facilitate repair (Hesse et al. 2012). CPCs on the other hand seem to differentiate in the border zone of injury to form new myocardial cells including CMs.

2.1 Limited regeneration in mammalian heart

Evidence of spontaneous processes that seem to repopulate heart tissue have gained much interest. Maintenance in homeostatic conditions involves slow cell renewal. On the other hand, injury seems to lead to minor repair.

In postnatal mammalian heart, formation of new cardiomyocytes has been recorded (Bergmann et al. 2009). At 25 years of age approximately one percent of human cardiomyocytes are replaced each year. This turnover rate decreases with age, being 0,45 % at 75 years of age. A different study suggests an annual CM turnover rate as high as 22 % (Kajstura et al. 2010). The wide range of these results highlights the difficulty of interpreting findings especially from single experiments in the field of regeneration research. Turnover rate of around one percent is widely accepted. The new cardiomyocytes originate from pre-existing ones (Senyo et al. 2013). Similar renewal of cardiac cells also takes place in mouse hearts with normal CM turnover of 1,3-4 % per year (Malliaras et al. 2013). This also results from the proliferation of mononucleated CMs with little to no contribution from CPCs. Contradictory results have been published suggesting that primarily CPC differentiation accounts for CM generation that maintains homeostasis (Hosoda et al. 2009).

Following cardiac injury, an amount of new CMs appear at the borders of the lesion but the CMs formed don´t result from CM proliferation (Malliaras et al. 2013; Hsieh et al. 2007). Only a small minority of CMs in the heart of an adult mammal can reenter the cell cycle, which prevents significant cardiac regeneration (Takeuchi 2014). In a model of MI the new cardiomyocytes appearing in the infarct border zone originate mostly from differentiated stem cells (Malliaras et al. 2013). Another study found no proliferating CMs in the border zone of injury (Hesse et al. 2012). They attributed DNA replication without cell division.

Heart has a heterogeneous population of multipotent cells called cardiac progenitor cells (CPCs) (Le and Chong 2016). The CPCs are able to self-renew and differentiate into CMs among other cell types of the heart (Hastings et al. 2015). This regenerative

potential is evident *in vitro* but seems to rarely occur *in vivo* under normal conditions. Hearts CPCs seem to take part in CM generation only after an insult (Malliaras et al. 2013). Mainly quiescent CPCs activate in response to injury and some new CMs are generated from the CPCs. The generation of myocardium in the border zone of injury is insufficient for functional repair, likely due to the small number of CPCs. Adult mammalian heart is capable of restoring lost cardiomyocytes in certain situations (Ellison et. al. 2013). All in all, processes of endogenous regeneration occur in the heart and this provides support for the notion of regenerative therapies that promote endogenous repair mechanisms.

2.2 Hearts embryonic development

Prospects for therapy arise from advancing understanding of how heart develops. Heart is the first organ to form in a developing embryo (Vliet et al. 2012). Early cardiogenesis has been examined in mouse model extensively, but the process is highly conserved so the findings apply to human development (Vincent and Buckingham 2010).

The programs that direct embryonic development can be translated to strategies of regeneration. In general, the transcriptional pathways involved during embryogenesis also mediate tissue regeneration (Uygur and Lee 2016). Transcription factors (TFs) and miRNAs have been used to activate pathways involved in cardiogenesis, improving damaged hearts function (Xin et al. 2013).

In mouse embryos cardiac precursor cells found in the epiblast ingress through the primitive streak during gastrulation (Vliet et al. 2012). They migrate to anterior-lateral direction at E6.5 to form two cell groups on both sides of the midline termed the cardiogenic fields (Xin et al. 2013). The cells in the first or primary heart field (FHF) subsequently fuse into cardiac crescent at E7.5 and form a heart tube by E8. Another group of cells called the secondary heart field (SHF), positioned within the pharyngeal mesoderm medial to FHF migrates to venous and arterial poles of the heart tube (Buckingham et al. 2005). The heart tube starts beating synchronously and goes through a looping process that gives rise to early atria and ventricles at around E8.5 (Xin et al.

2013). After the looping of the heart tube, distinct chambers emerge by E10.5 (Buckingham et al. 2005). Septation separates the chambers by E14.5.

Majority of hearts cells, including all of myocardium, originate from the cardiogenic fields in the lateral plate mesoderm. FHF descendants form the left ventricle and part of the right ventricle, whereas rest of the right ventricle derives from SHF cells (Hartogh and Passier 2016). Both heart fields' cells make up the atria. A minor proportion of the hearts tissues originate from anterior neural crest (Vincent and Buckingham 2010). Cells from the cardiac neural crest contribute to aorta and pulmonary artery, hearts valves, septum, conduction tissue (Vincent and Buckingham 2010).

2.3 Signaling during early cardiogenesis

Heart induction is controlled by a complex system of signaling pathways in a process that is very similar amongst vertebrates (Liu and Foley 2011). Earliest precursors of cardiac lineage reside in epiblast at pre-streak stage, E6.0 in mice (Vliet et al. 2012). These cardiac precursors are among the subsets of epiblast cells to assemble to midline where primitive streak forms (Santini et al. 2016). The primitive streak (PS) formation starts the gastrulation process that forms three embryonic germ layers. PS forms as nodal is produced by node. PS development is controlled by Wnt5 and Wnt3a produced within the streak. Progenitor cells in the epiblast undergo epiblast-mesoderm transition (EMT) as they ingress through the primitive streak and establish mesendoderm (Vliet et al. 2012). First ingressing cells form visceral endoderm and the next come to form mesoderm, but while migrating they temporarily constitute mesendoderm (Vliet et al. 2012).

The first cardiac precursor cells become specified for their fate upon leaving the primitive streak for prospective cardiac mesoderm (Paige et al. 2015). Progenitor cells' lineage potency decreases as they ingress through the primitive streak (Tam et al. 1997). Early cardiac progenitor development from epiblast to mesendoderm depends on Wnt, Nodal and BMP pathway signals (Laflamme and Murry 2011). Whereas combination of

canonical Wnt and nodal signals is needed for mesendoderm formation, BMP2 plays a role in establishing cardiogenic region from mesendoderm (Vliet et al. 2012).

Mesp1 gene expression drives cardiac specification and functions as a kind of master gene in cardiac development (Vliet et al. 2012). Cardiac progenitor differentiation begins with MesP1 TF expression, caused by eomesodermin at the primitive streak (Paige et al. 2015). Mesp1 takes part in EMT and activates central cardiac TFs driving cardiac specification (Vincent and Buckingham 2010). It´s expression fades as the progenitor cells migrate to heart forming region (Paige et al. 2015).

While FHF and SHF originate from the same progenitor, the lineages segregate at the beginning of gastrulation (Santini et al. 2016). SHF progenitors differentiate later. Cardiac lineage develops through stages marked by different genes: mesendoderm progenitors express *brachyury*, and mesodermal ones express Mesp1, Flk1 and PDGFRα (Willems et al. 2011). Nkx2.5 marks progenitors of the cardiac crescent and finally CMs exclusively express myosin chain genes. Tbx5 distinquishes FHF and Tbx1 SHF progenitors. Interestingly, Islet1 (Isl1) is much expressed in proliferating SHF but is inactivated by Nkx2.5, triggering differentiation as the cells join the heart tube (Calderon et al. 2016).

Progenitor commitment to cardiac phenotype is dependent on inductive signaling in the heart forming region (Tam et al. 1997). The cardiac progenitors reside under the neural plate and adjacent to visceral endoderm underneath at around E7.0, the late PS stage (Vliet et al. 2012). Endoderm holds an essential role in determining cardiac fate through paracrine signaling (Calderon et al. 2016). Anterior primitive endoderm promotes cardiac fate with Shh and FGF factors, Wnt inhibitors and BMP (Paige et al. 2015). Neural plate produces canonical Wnt pathway activators and notochord BMP inhibitors that restrict differentiation towards cardiac cells (Xin et al. 2013). This regulation serves to limit cardiac mesoderm formation to where it’s supposed to take place (Paige et al. 2015).

SHF progenitors also require external signals to maintain progenitor phenotype and proliferate (Paige et al. 2015). Situated in pharyngeal mesoderm, SHF proliferation is encouraged by Shh, which is produced by adjacent pharyngeal endoderm. FGF3, FGF8 and FGF10 present in surrounding mesoderm as well as canonical Wnt activity direct progenitor development.

2.4 In vitro differentiation

Development of ESCs into cardiac lineage cells and CMs *in vitro* reflects the process of early cardiogenesis *in vivo* (Kurosawa 2007). The same key growth factors control differentiation of pluripotent cells towards cardiac fate. The differentiating cells develop into consecutive progenitors that represent the stages of embryonic development (Vliet et al. 2012). ESCs are blastocyst cells and develop through epiblast stage precursors to mesendodermal progenitors to mesoderm and cardiac mesoderm stage that gives rise to early CMs. In line with this, both FHF and SHF progenitors along with their respective lineages are spontaneously formed by differentiating ESCs. Still, plasticity of mESCs might allow cell development to proceed through ways that it wouldn't *in vivo* (Vliet et al. 2012). The process of ESC differentiation down the cardiac lineage is shown in figure 1.

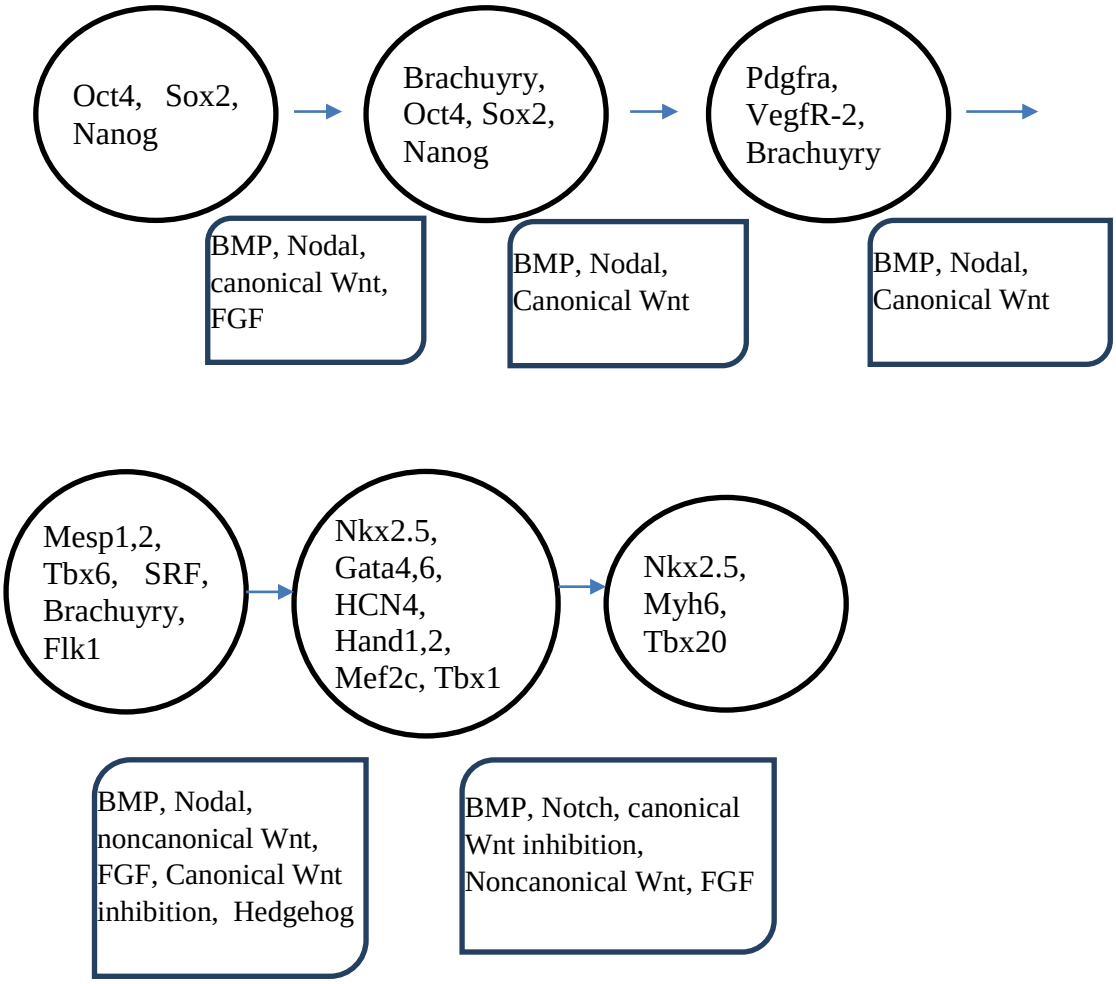

Figure 1. mESC differentiation. Major signals promoting specification and differentiation at each stage are presented (Noseda et al. 2011; Laflamme and Murry 2011; Sahara et al. 2015).

Inductive influence of endoderm is equally critical for determination of mESCs to differentiate towards CM fate (Vliet et al. 2012). Presence of endodermal cells or their secretome promotes cardiac differentiation, with BMP2 as primary inductive factor. Nodal and BMP signals are interdependent.

Proliferative and differentiation inducing signals are largely opposite at CPC stage (Sahara et al. 2015). For instance canonical Wnt signalling induces cardiac lineage in early stage and hinders it at later stages (Uosaki and Yamashita 2011). While

differentiation of CPCs requires inhibition, canonical Wnt plays a central role in proliferation and maintenance of the same progenitors.

Variety of protocols has been established for promoting efficient differentiation of mESCs into CMs (Kokkinopoulos et al. 2016). Majority of these techniques depend on formation of spontaneously differentiating cell aggregates called embryonic bodies (EBs). Some alternative protocols exist with cells grown in monolayer format. First strategies of inducing cardiac cells from ESCs were based on Wnt antagonists (Alamo et al. 2016). Dkk1 protein and small molecules were used, resulting in 10-50% CM yield. Ascorbic acid was the first molecule described to improve differentiation of ESCs to CMs (Takahashi et al. 2003). Also serum free conditions are typically used to enhance cardiac differentiation (Hartman et al. 2014). The methods for generating CMs from ESCs are typically complex and involve challenging practices (Hartman et al. 2014).

3 Regenerative strategies

Ischemic injury affects all cells in the affected area of the heart, which consists of a number of cell types with specific functions. Cardiomyocytes are especially susceptible to the lack of oxygen. This is why strategies to repair myocardium mainly aim to restore CMs and/or vasculature. Cell-based methods involve transplantation of cells from exogenous source into the myocardial tissue (Sahara et al. 2015). Cell therapy with CMs cultured *in vitro* and introducing CPCs are examples of cell-based therapy. Research into cell-free strategies is aimed at fibroblast reprogramming, endogenous CPC mobilization or stimulating CM proliferation (Bruneau 2013).

3.1 Enhancing proliferation of CMs

Species such as zebrafish have been studied in order to understand the features that allow hearts spontaneous regeneration in lower vertebrae. Zebrafish have an ability to functionally recover from extensive injuries to heart tissue (Jazwinska and Sallin 2015).

In the process of regeneration the zebrafish CMs dedifferentiate to a more primitive state, which allows for cell division while retaining some contractile function (Kikuchi and Poss 2012). The cardioregerative ability of zebrafish among lower vertebrates has been linked to lower overall metabolic state, cardiac workload and lesser dependency on coronary circulation compared to mammals (Jazwinska and Sallin 2015). Cardiac regeneration in neonatal mice proceeds through proliferation of the existing CMs as well (Porrello et al. 2011). Elaborate structure with thick compact myocardium and system of coronary vessels may hinder regenerative response to insult in postnatal mammals (Kikuchi and Poss 2012).

Like zebrafish, neonatal mice and humans can recover from heart injury through CM proliferation (Kikuchi et al. 2010). Mammalian heart's regenerative potential diminishes shortly after birth (Bruneau 2013). Human CMs withdraw from cell cycle in childhood (Mollova et al. 2013). Still, adult mammalian heart may have capacity for regeneration that could be revived (Bruneau 2013). To stimulate CMs cell cycle re-entry would leverage an existing regeneration mechanism (Bruneau 2013). Transcription factors, microRNAs and small compounds have been demonstrated to modestly stimulate the proliferation of adult CMs even though the molecular mechanisms causing cells cycle arrest in mature CMs are only partially understood (Ahuja et al. 2007). An increase in proliferation of adult CMs has been demonstrated by regulation of gene expression with miR-590 or miR-199a both *in vivo and in vitro* (Eulalio et al. 2012). Similarly, repair of myocardial tissue has been activated by introducing paracrine factors such as VEGF, TB4 or Neuregulin 1 (Bersell et al. 2009; Karathanasis et al. 2014). Functional improvements are modest, but combinations of several factors have been proposed to further increase the positive results.

One approach for spawning CMs is to reprogram other cells types within the heart (Karathanasis 2014). Heart's excess fibroblasts represent a lucrative cell type to convert. Direct reprogramming of these cells has been demonstrated by activating specific regulatory systems with TFs, miRNAs or synthetic compounds. However, the issue of low conversion efficiency still remains. *In vivo* environment may favor differentiation better than *in vitro* culture (Bruneau 2013).

3.2 Endogenous CPCs

Heart's endogenous CPCs are multipotent and have the potential to generate most of the cell types necessary for cardiac regeneration *in vitro* (Schade and Plowright 2015). Whether heart's CPCs generate new functional CMs *in vivo* remains a matter of debate with convincing evidence published both for and against this notion (Cesselli at al. 2017). Molecular mechanisms that guide proliferation and differentiation of these CPCs remain largely unknown (Drowley et al. 2016). Enhancing proliferation and differentiation of CPCs represents an interesting strategy to regenerate myocardium *in situ* (Schade and Plowright 2015).

Existence of CPCs in adult mammalian heart is well established but identity and specific biological roles of CPCs remain unclear (Le and Chong 2016). These cells are heterogeneous and reside in special niches spread around the heart (Broughton and Sussman 2015). CPCs are estimated to make up 0,005-2% of adult hearts cells and take part in maintaining cardiac homeostasis (Santini et al. 2016; Schade and Plowright 2015). These CPCs suppress apoptosis of CMs and activate many types of heart's cells to repair through paracrine signaling (Schade and Plowright 2015). What defines the pool of endogenous CPCs is their potential to re-enter cell cycle, proliferate and differentiate into several cell types, albeit this might not occur *in vivo* (Hastings et al. 2015). Different populations with this potential have been characterized based on certain markers and development of isolated cells grown *in vitro*, but none of the markers is unique to heart's progenitors (Le and Chong 2016). These subpopulations of CPCs are not well defined and overlap in gene expression (Cesselli at al. 2017).

Some examples of defined adult CPC populations reported include c-Kit+ (KIT), Sca-1+, Isl1+ and cardiosphere derived cells (Le and Chong 2016). Isl-1 has since been found to not mark adult CPCs. The population of KIT (also c-kit or CD117) positive CPCs was the first one discovered and likely the best studied population (Koudstaal et al. 2013). KIT expression has been proposed to restrict cell proliferation and defines a mixed population that contains resident progenitors but also cells from bone marrow

and non-stem cells (Santini et al. 2016). KIT progenitors are rare in adult heart. Stem cell antigen 1 (Sca-1+) marks a very heterogeneous group of heart's cells, some of which are progenitors. Bmi1+ CPCs presumably make up a subpopulation of Sca-1+ progenitors (Valiente-Alandi et al. 2016). Expression of Bmi1 protein mediates proliferation of many adult stem cell populations throughout body. CPCs found within heart may be residual cells from embryonic development (Zelarayan et al. 2016).

CPCs have been reported to be able to replace CMs after diffuse myocardial damage in adult rodent heart (Hsieh et al. 2007). According to study by Ellison et al. the stem cell niche expressing KIT marker proliferates and the progeny differentiates into functional cardiomyocytes in response to diffuse myocardial damage (Ellison et al. 2013). Contribution of other cell types wasn't ruled out, but KIT population was identified to account for majority of the spontaneous CM replenishment. The reported CM replacement is quite extensive, accounting for several percent of heart's total CMs. In the case of MI however, the affected myocardium clearly doesn´t regenerate on its own. Contradicting the above, KIT CPCs are also reported to only form insignificant amount of new CMs in several more recent studies even in the same model of diffuse myocardial damage as used by Ellison group (Valiente-Alandi et al. 2016; van Berlo et al. 2014). Recent study suggests that c-kit+ CPCs in developing and adult heart originate from cardiac neural crest, while adult heart may also contain c-kit+ cells of other origins (Hatzistergos et al. 2015). BMP signaling was found to be central factor in suppressing their cardiomyogenic differentiation. Evidently, differentiation of the endogenous CPCs *in vivo* may be restricted by cardiac environment, since these cells are capable of symmetric or asymmetric division and differentiation *in vitro* (Cesselli at al. 2017). Accumulating knowledge of the mechanisms involved and well established presence in mammalian heart makes KIT a reasonable target population for therapeutics (Hatzistergos et al. 2015).

Other CPC populations of adult heart capable of *de novo* CM generation have also been described. CPCs with high expression of Bmi1 reportedly proliferate and form CMs in adult mouse heart after MI. (Valiente-Alandi et al. 2016). These CMs form in the border

zone of injury and are similar to adult CMs unlike the progeny of other CPC populations.

Cell therapy with KIT and cardiosphere derived CPCs have reportedly improved cardiac function after myocardial damage in clinical studies (Santini et al. 2016). Other CPC subpopulations and other stem cells have presented similar positive effects when implanted into animal heart. The improvement has been attributed to paracrine effects and vasculogenesis, as no significant CM regeneration is reported. Effective pharmacological therapies might require ways to combine proliferative effect with inducing differentiation. This would be to avoid exhausting the small pool of progenitors present in the heart.

3.3 Model for cardiac tissue

Progenitor cells resembling CPCs of adult heart (eCPCs) can be obtained from ESCs. Screening systems based on ESCs are frequently used as a model to look for small molecules or for example microRNAs that would drive CPCs to produce cardiomyocytes and other cardiovascular cells (Spiering et al. 2015).

Phenotypic screening approaches have yielded majority of first-in-class drugs approved for use in recent years (Swinney and Anthony, 2011). Phenotypic assays identify effects on a signalling pathway of interest. This effect is visualized and quantified by expression of a fluorescent or luriferase reporter. This approach is typically chosen when probing for a specific response, but the exact signalling pathway or binding site for putative drugs are unknown. The desired cellular response can arise from any number of targets that provoke it.

The use of a reporter line has multiple benefits in screening (Schade and Plowright 2015). Assay development is facilitated as reporter expression can be followed in real time. Assay controls are immediately visible before processing the plates. Antibody-based read outs are costly and add extra steps.

3.4 Strategy of using small molecules

Among potential means for treatment of heart failure and loss of viable myocardial tissue, small molecules are especially attractive and could become future pharmaceuticals for promoting regenerative heart repair (Russell et al. 2012). Synthetic drugs are generally relatively inexpensive to produce and their pharmacokinetic and -dynamic qualities can be tweaked for optimal effects (Schade and Plowright 2015). Such molecules can also pass through cell membranes (Längle et al. 2014). Acellular therapies in general are likely more straightforward to translate into clinical treatments due to not depending on a source of cells (Hastings et al. 2015). Small compounds also have a consistent effect and may regulate several targets and mechanisms. Numerous compounds that activate CM proliferation or enhance formation of CMs from CPCs have been discovered (Längle et al. 2014). Some compounds were first found for cancer research and affect major developmental pathways (Schade and Plowright 2015). Few compounds stimulate formation of CMs and tissue repair *in vivo* (Schade and Plowright 2015).

Canonical Wnt pathway is a complex system of signals with a key role in development but also at later stages of life (Lenz and Kahn 2014). The pathway has functions in all organ systems. Controlling canonical Wnt pathway has been identified to promote cardiac fate most strongly in uncommitted CPCs (Schade and Plowright 2015). Many compounds and their structure activity relationship have been extensively studied. Tankyrase targets have sparked interest as a target for inhibition in pharmaceutical industry. To address the inherent safety issues that arise from influencing a major developmental pathway, compounds targeting downstream effectors of Wnt/β-catenin are being developed. PRI-724 and ICG-001 are such molecules, inhibitor of CREB binding protein (CBP)/ β-catenin selectively blocks transcription mediated by that complex. ICG-001 is actually not toxic and it drives CPC differentiation (Längle et al. 2014).

Selective cannabinoid receptor 2 agonist AM1241 improves cardiac function after MI possibly by stimulating CPC proliferation (Schade and Plowright 2015). Prostaglandin

E2 (PGE2) causes differentiation of Sca-1+ CPCs to CMs *in vivo*. These may however result from indirect effects on the environment.

Several agents that increase proliferation of CMs have been discovered as well (Schade and Plowright 2015). Inhibitor of p38MAPK and GSK3β inhibitor boost CM regeneration in adult rodent myocardium after MI. GSK3β inhibition has an effect similar to Wnt activation. This inhibition is downstream of Wnt pathway signal cascade. CaMK2 inhibitor and ERK activator (like NRG1β), which only seem have an influence on early CMs are pro-proliferative *in vitro* but not in adult heart *in vivo* (Uosaki et al. 2013). Similarly, periostin ligand also promotes CM proliferation increasing cardiac function following an injury (Sahara et al. 2015).

More basic research has helped in finding additional pathways that seem to play a role in regulation of CM proliferation. Many potential targets for future drug discovery have been identified within these pathways. One such case is Meis1 transcription factor that apparently downregulates several cyclins in CMs and it's inactivation could prolong the period of postnatal proliferative capability of CMs *in vivo* (Schade and Plowright 2015). Another example is Hippo pathway inactivation that results in increased CM proliferation *in vivo* (Schade and Plowright 2015). Dedifferentiation of CMs is associated with proliferation as well (Zelarayan et al. 2016). For example, oncostatin M may take part in dedifferentiation.

Pharmacological therapy has great potential demonstrated by variety of ways *in situ* repair could be induced. More potent, safer compounds are needed. The physicochemical properties of each test compound need to be determined at the beginning of preclinical testing, since they determine its utility. Unsatisfactory pharmacokinetic attributes are the most prominent reason for abandoning a drug at a clinical phase. Solubility in gastrointestinal system, stability, logP, logD, permeation trough cell membranes and the effect of efflux proteins are assessed *in vitro* early on. *In vivo* or *ex vivo* testing is needed to establish absorption, distribution, metabolism and excretion of a candidate drug.

Molecular mechanisms that guide specification into ventricular, atrial or other subtypes of CMs are also of interest (Schade and Plowright 2015). For example, treatment with 1-EBIO generates a culture of CMs with mostly pacemaker-like phenotype. 1-EBIO activates calcium dependent potassium channels. AG1478, an ErbB antagonist has a similar effect to the phenotype. Retinoic acid has potential to strongly favor atrial phenotype, while addition of RA receptor antagonist BMS-189453 may produce more ventricular subtype. Inhibition of canonical Wnt pathway seem to generate more ventricular CMs from hPSCs. Wnt pathway appears to be a central component in subtype specification.

3.5 mESC line used for screening

Murine embryonic stem cell lines that originate from E14 were used for the assay. The lines' Myl2-eGFP reporter gene facilitates identifying and quantifying the ventricular cardiomyocytes. Ventricular myosin light chain 2, or for short Myl2 gene is specifically expressed in heart's ventricular myocytes (Hartogh and Passier 2016). The Myl2 protein and the adjacent eGFP make up a part of sarcomeres so the reporter expression starts in immature cardiomyocytes. Cardiomyocytes mature trough increasing their sarcomere content and thus eGFP reporter intensity increases with maturation. A double reporter mESC line carrying an additional atrial Smyhc3-RFP marker is utilized in the developed assay. Slow myosin heavy chain 3 (Smyhc3) promoter transgene, specific to atrial CMs allows recognition of chamber subtypes through RFP or eGFP labels, respectively (Wang et al. 1996).

4. Aim of the study

The aim of this study was to develop a screening method for small compounds that enhance cardiac progenitor or stem cell proliferation and/or differentiation. The mESC Myl2-eGFP reporter lines had been used for drug discovery assays previously, so the existing methods to acquire and handle the cells were used as a basis for assay design. Central goal was to facilitate sufficiently high throughput to test possibly hundreds of

conditions at once. First objective was to utilize an established differentiation protocol to set up a pilot assay in 96 well format. Next was to miniaturize the assay and optimize data quality and practicality. Another goal was to validate the assay using a small number of selected compounds.

5. Materials and methods

The mESCs used were Myl2v-eGFP reporter lines, derived from E14 mESC line. The parent wild type line was used as a control. The cardiac progenitor cells needed for the screening are obtained from mESCs via directed differentiation. The differentiation technique used is based on protocol by Kattman et al. in 2011. The pluripotent mESCs are first grown in conditions inhibiting differentiation. Differentiation is provoked by transferring the mESCs to neutral conditions where they spontaneously form embryonic bodies (EBs). After two days of initial culture, a specific set of cytokines is introduced in order to quide specification of the cells towards cardiac lineage. By day 4 early cardiovascular progenitors expressing PdgfR-α and Flk-1 are present (Kattman et al. 2011). These CPCs continue to develop dependent on conditions and can be steered to become CMs by day 7 or 8.

5.1 Cell culturing

Cells of all stages were grown at 37℃ under atmosphere with 5% CO_2 and high relative humidity.

Collecting mESCs from a T25 flask: Medium is aspirated and the cell layer washed with 7 ml of PBS. 1 ml of TrypLE is added and the flask incubated in 37℃ for 4 minutes to detach the cell layer. Clumps of cells are broken up through repeated pipetting and 1 ml of MEF medium is added to inactivate TrypLE. The suspension is transferred into a tube with 8 ml of MEF medium. The tube is centrifuged at 1200 RPM for 3 minutes and the supernatant aspirated.

Washing steps: 10ml of IMDM is added onto cell pellet in a centrifuge tube, centrifuged at 1200RPM for 3 minutes and the supernatant aspired.

5.2 Media

Media components used for differentiation are presented here.

Media	**Details**	**Supplier catalog number**	**Brand/Supplier**
PBS	Without Ca++, Mg++ or phenol red	BE17-516F	BioWhittaker Lonza
DMEM	with 4,5 g/l glucose, without L-Glutamine	BE12-614F	BioWhittaker Lonza
FBS	Fetal Bovine Serum	10270-106	Gibco® Life Technologies
SP34	StemPro®-34 Serum-free medium kit: SP34 medium and nutrient supplement	10639-011	Gibco® Life Technologies
IMDM	without L-Glutamine, with sodium bicarbonate	I3390	Sigma-Aldrich Life science
Ham's F12	with L-Glutamine	10-080-CVR	Corning
Embryomax FBS	Fetal Bovine Serum, ESC quality	ES-009-B	EmbryoMax® Millipore
GlutaMAX	100X L-Glutamine in stabilized form	35050-038	Gibco® Life Technologies
MEM NEAA	100X Non-essential amino acids	11140-035	Gibco® Life Technologies
2-Mercaptoethanol	1000X, 55mM in DPBS	21985-023	Gibco® Life Technologies
MTG	1-Thioglycerol >97% for cell culture	M-6145	Sigma-Aldrich Life science
N-2	100X N-2 supplement	17502-048	Gibco® Life Technologies
B-27	50X B-27 supplement, minus vitamin A	12587-010	Gibco® Life Technologies
0.1% Gelatin	0.1% porcine gelatin in water	ES-006-B	EmbryoMax® Millipore
TrypLE	1X TrypLE Express enzyme, without phenol red	12604-013	Gibco® Life Technologies

Cytokines	Details	Supplier catalog number	Brand/Supplier
LIF	Mouse leukemia inhibitory factor 10^7 units/ml	ESG1107	Esgro® Millipore
BMP4	Bone morphogenetic protein 4, recombinant human	314-BP-010	R&D Systems
Activin A	Recombinant human/mouse/rat activin A protein	338-AC-010	R&D Systems
FGF10	Fibroblast growth factor 10, recombinant human	345-FG-025	R&D Systems
hFGFb	Fibroblast growth factor basic, recombinant human	233-FB-025	R&D Systems
hVEGF	Vascular endothelial growth factor	293-VE-010	R&D Systems

Commonly used media are presented here.

MEF medium: DMEM + 10% FBS

ES medium: DMEM, 15% Embryomax, 1% Glutamax, 1% MEM NEAA, 0.1% 2-mercaptoethanol, 0.01% LIF

SP34: StemPro34 SFM 50ml, SP34 nutrient supplement 1.3ml, Glutamax 500µl

SFD: IMDM, Ham's F12 25%, 10% BSA in PBS 0,5%, Glutamax 0.75%, B27 1%, N2 0,5%

Maintenance medium (MM): SP34 (With supplement and glutamax), VEGF 5ng/ml, FGFb 10ng/ml, FGF10 25ng/ml, Ascorbic acid 50µg/ml

5.3 mESC culture

At the initial pluripotent stage, mESCs are grown in feeder free cell culture with leukocyte inhitory factor (LIF) included in the medium, in adherent conditions.

Mouse ESCs are cryopreserved at -160℃ divided into small vials that make up the stock. To start each experiment, a cryovial is thawed and the cells cultured for four days (figure 2). The mESCs are cultured in LIF containing ES medium to maintain undifferentiated state. During this period the cells propagate enough for sufficient cell

numbers for the assay. The growth period also serves to confirm pluripotency and the highly proliferative mESCs outgrow differentiated cells. Pluripotent cells are small and round, and the cells seem to visibly change shape when differentiating. Visual appearance can be used as a rough indicator for potency of the culture.

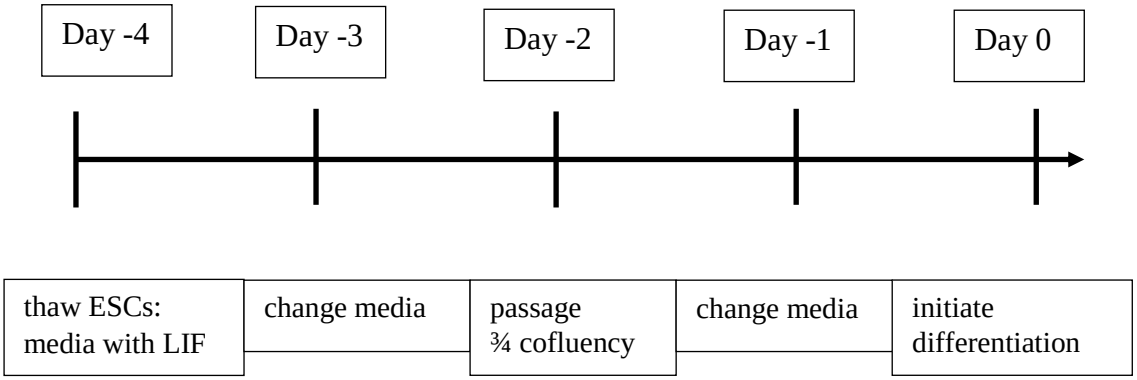

Figure 2. mESC culture timeline. The cells are thawed four days prior to beginning differentiation and grown in LIF containing medium until then. The medium is changed each day and cell density maintained by dividing at day -2 when close to confluency.

A vial of cryopreserved mESCs is transferred from cryotank to cell lab on ice and quickly thawed in 37℃ waterbath for 3 minutes. The cell suspension is diluted with nine times its volume of DMEM with 10% FBS, centrifuged and the supernatant aspired. The cells are resuspended and dispersed in around 8 ml of ES medium in a T25 flask coated with 0.1% gelatin. The cells are frozen in 10% DMSO containing medium. After thawing in the afternoon, ES medium is replaced early next day to remove any dead cells and residual DMSO.

The flasks are subcultured at day -2 to maintain subconfluency and the media changed daily. Subculturing is performed as follows: Medium is aspirated and the cell layer washed with 7 ml of PBS. 1 ml of TrypLE is added and the flask incubated in 37℃ for 4 minutes to detach the cell layer. Clumps of cells are broken up through repeated pipetting and transferred into a tube with 9 ml of MEF medium. The tube is centrifuged at 1200 RPM for 3 minutes and the supernatant aspirated. The pellet of cells is resuspended in an appropriate amount of ES medium and counted. 350 000 mESCs are seeded per T25 flask as described above.

5.4 Induction of cardiac mesoderm

The cells are cultured in suspension where they form EBs for four days to direct differentiation. For the first 48 hours of differentiation medium contains SFD with ascorbic acid and MTG. At day 2 cytokines are introduced and for 42 hours induction medium contains SFD with 5mg/ml ascorbic acid, 39nl/ml MTG, 5ng/ml VEGF, 7ng/ml Activin A and 0,7ng/ml BMP4.

Day 0 cells are collected from T25 flasks as described before. After aspirating MEF medium, the mESCs are washed twice with IMDM in order to remove serum and LIF. The resulting cell pellet is resuspended in 1 ml of IMDM for counting the cell concentration. These cells are then plated to 90mm dishes (Sterilin petri dish 90mm, Thermo scientific) in serum free differentiation media at 750 000 cells/10 ml per plate.

At day 2, the EBs are collected by transferring them into 15 ml tube with their medium using a serological pipette with a wide tip and washing the plate with 5 ml of IMDM while tilting the dish. The tube is centrifuged at 800 RPM for 3 minutes and the supernatant removed. The cells are then dissociated with addition of 1 ml TrypLE for 1,5 minutes in 37℃ water bath. After breaking the remaining clumps by pipetting, 9 ml of MEF is quickly added and the tube centrifuged at 1200 RPM for 3 min followed by washing twice with IMDM. The cells are counted and plated in the induction medium as on day 0.

Day 4 cells are handled like on day 2 and transferred onto ultralow attachment plates. 150 000 cells in 250µl of maintenance medium (MM) per well are plated on 24-well plates.

5.5 CPC Differentiation

The cardiospheres that have formed overnight are dissociated and plated onto screening plates (Corning® 384 well Flat Clear Bottom Black Polystyrene TC-Treated

Microplates, Sterile, # 3764) at day 5. The spheres are collected into a tube with 10 ml of PBS. They'll settle to the bottom in three minutes so that PBS can be aspirated. Miltenyi neonatal heart dissociation kit is used for dissossiation according to manufacturer's instructions. The plates are coated with 0.1% gelatin for 1 hour at room temperature. 10 000 cells in 50µl of maintenance medium are plated per well on 384 well plates. The plating is done as fast as possible with multichannel pipette and the cells are allowed to settle in the hood undisturbed for 20 minutes before moving the plates into incubator. Two outermost wells were not used in the experiments to avoid edge effects. They were filled with PBS and utilized as blanks.

To study effects in CPCs, medium is changed to one with compounds to be tested at day 6. Compounds are added to maintenance medium and since they come dissolved in DMSO, 0.1% DMSO is used as vehicle. Four technical replicate wells were created. Medium is changed back to MM at day 8. For effect at even later stage, the same molecules were added to another set of wells were treated day 7-9. The medium was changed daily. As the positive controls 3, 10mM XAV939 (Sigma-Aldrich) is used. Figure 3 illustrates the timeline of compound treatments.

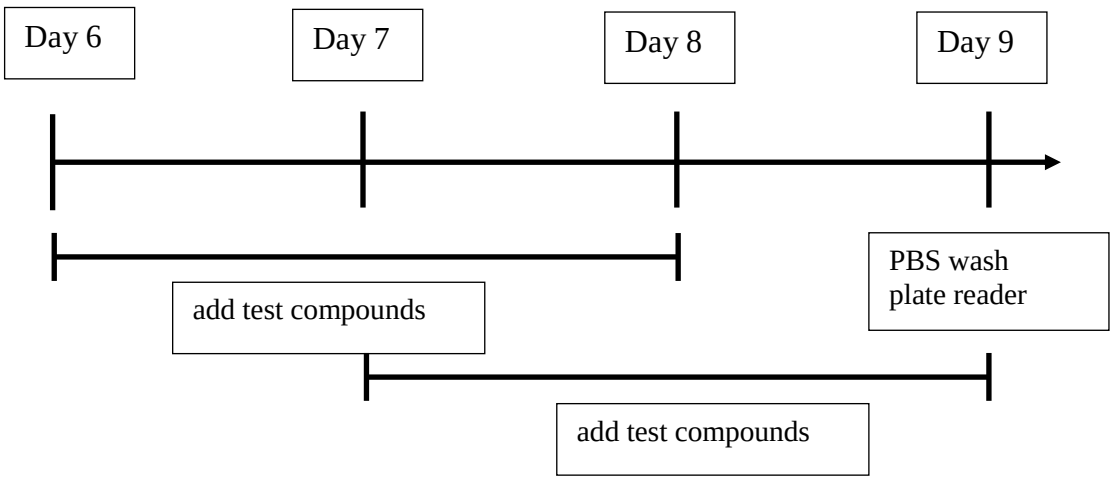

Figure 3. CPCs were treated with test compounds day 6-8 and other cells day 7-9. The CPCs reach early CM stage in their development by around late day 7, so the later treatment schedule gives information about effects towards primitive CMs rather than CPCs.

5.6 Total fluorescence quantification with plate reader

Total fluorescence of the whole cell population in each well was measured to assess the effect of different treatments. Assay endpoint was total fluorescence, which was measured at day 9 of the experiments. The plates were washed 4-5 times with cold PBS to remove the media and placed on ice until reading. Pherastar FS reader at 485/520 nm with bottom reading was used. With orbital averaging the reader measures 20 spots around the well and provides the average of these as a result.

To account for background fluorescence, blank wells were placed on each plate. Background was subtracted from raw values. Wild type cells had the same fluorescence as the empty with just PBS. Interestingly, wells with just media without cells had higher BG fluorescence even after multiple washings.

5.7 Flow cytometry

BD Accuri C6 Sampler was used to quantify cells at different stages. Laser based flow cytometry technology allows counting and characterizing individual cells in a suspension based on their physical features (Jaroszeski and Radcliff 1999). Ventricular CMs are identified by the endogenous Myl2-eGFP marker. cTnT staining marks all CMs. The instrument provides measurements of each cells several traits, making the overall result highly accurate. Flow cytometry was used as a reference method and to inform adjustment of cytokine concentrations for induction stage.

5.8 Method optimization

Different numbers of cells were plated on the 384 well plates at days 5, 6, 7 and 8 to establish optimal conditions for the screening. Media with different growth factors were tested for each day and cell number. Wnt inhibitor XAV39 was used as a model compound to produce strong positive effect compared to the DMSO control.

5.8 Immunofluorescent staining of adherent cells

Cell populations on plates and coverslips were fixed and stained for various intracellular markers in order to further characterize them. The cells were fixed with 4% PFA solution for 15 minutes, permeabilized with 0,5% Triton-X 100 for 15 minutes. Treatment with 3% BSA for an hour at 20℃ was used for blocking. Samples were exposed to primary or conjugated antibody in 3% BSA at 4℃ overnight, and to secondary in plain PBS for an hour at 20℃. Vectashield with DAPI (VECTASHIELD Antifade Mounting Medium with DAPI, Vector laboratories #H-1200) was used for nuclei stain.

6. Results

Developed assay was to identify treatments that stimulate CPCs' ability to proliferate and differentiate into CMs. The Myl2-eGFP marker of the reporter line used confirms the presence of ventricular CMs and allows quantification of these cells in a straightforward manner. Acceptable separation of positive and negative signals was achieved and treatments affect the readout signal enough to unambiguously tell apart positive, negative and baseline as illustrated in figure 4.

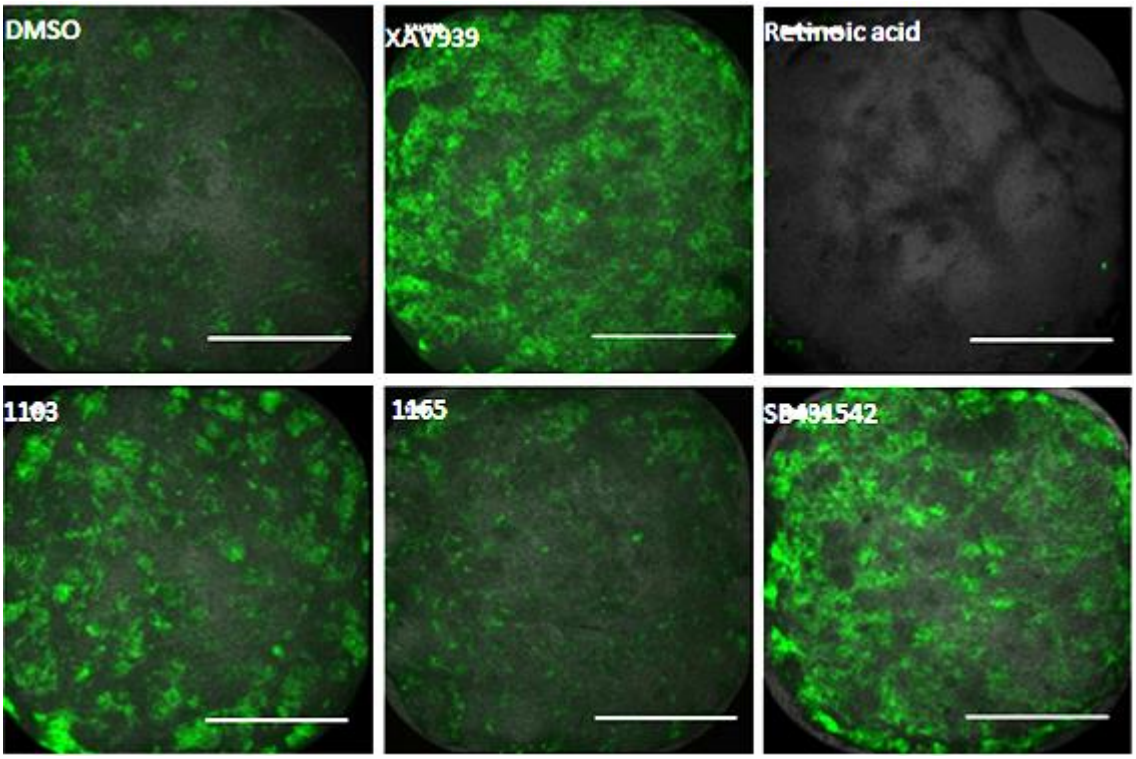

Figure 4. Collection of wells representative of an assay with compounds added day 6-8. Retinoic acid was included as negative control and XAV939 as positive control with 0,1% DMSO as vehicle. White scale bar is 1mm in length.

6.1 Assay design

The phenotypic assay design provides an unbiased way to determine effects of applied treatments. Total green fluorescence corresponding to the amount of myl2-eGFP protein was used as the assay endpoint to indicate the biological effect. This assay design inherently doesn´t discriminate between CM number and an increase in Myl2 expression. Myl2 is a sarcomeric protein so it´s expression in CMs increases with maturation process. Positive control XAV939 promotes CPC differentiation to ventricular CMs and also increases Myl2 expression. Retinoic acid was included as negative control. A small set of other molecules was used for testing during assay development. A few of the other compounds were also known to affect cardiac lineage differentiation.

Ventricular CMs identified by Myl2 constitute major part of all CMs *in vivo* as well as within the *in vitro* populations that form on the assay plates. Other subtypes of cardiac myocytes include atrial and conductive system cells (Sahara et al. 2015). Atrial CMs can be identified separately by their respective SMyHC3-RFP marker that specifically distinguishes the phenotype. Reading the second signal of red fluorescence at 540/590nm along with the quantification of the primary marker GFP presents no need for additional effort. To verify that CMs appear on the plates, cells derived from the model stained positive for several cardiac markers (figure 5). Additionally a widely used marker for CMs, cardiac troponin T (cTnT) is expressed in Myl2v+ cells as illustrated. Spontaneously beating regions visible under microscope also appear on the plates. The patches of beating cells align with situation of GFP fluorescence. Cells with CM phenotype appear between day 7 and 8 and continue to mature during the following days.

Assay design provides an opportunity to add the test compounds at a later stage so a variation of the first method with treatment days 7-9 was developed simultaneously. With at least part of the CPCs adopting early CM identity by late day 7, data from these experiments could point out regulators more relevant to that stage in development. This

assay variant doesn't include positive or negative controls and isn´t functional as a standalone assay as is.

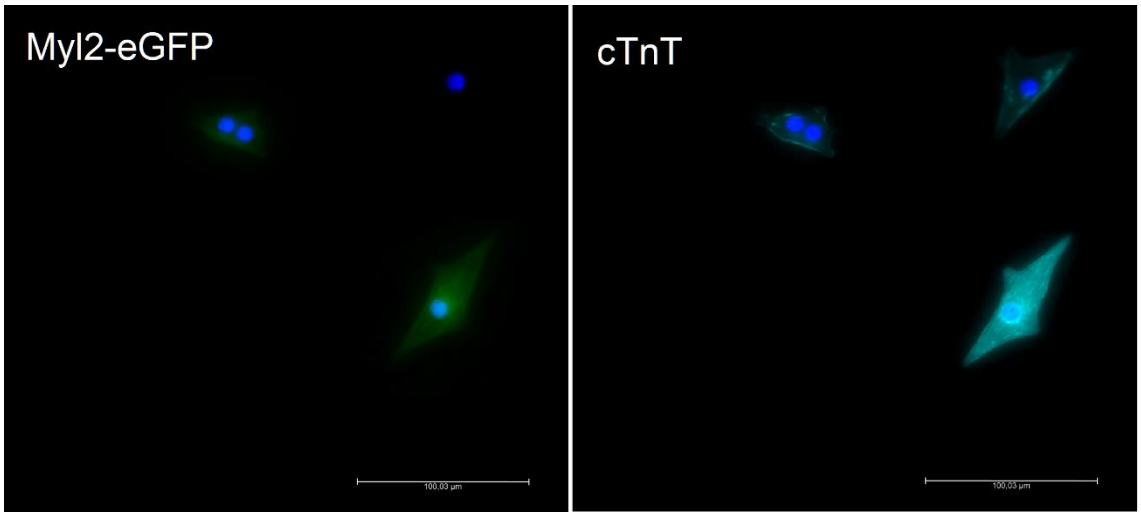

Figure 5. Cells positive for cTnT at day 20. Cells used for the assay differentiate into cardiomyocytes with cardiac markers. Myl2 positive cells also stain positive for cTnT. Scale bar = 100µm

6.2 Development and optimization

Miniatyrization from 96 to 384 well format didn't affect cell growth, but number of technical replicates was increased to four due to increased technical difficulty and error rate. Wild type cells were plated as blank controls for initial experiments. Autofluorescence of these cells was found negligible and equal to empty wells. Instead, media residue is strongly fluorescent at green wavelength and significantly contributes to well-to-well variability.

The yield of CMs in the neutral control wells was found highly variable between differentiations and plates when setting up the assay. Pluripotency of the starting mESCs was confirmed through staining for Oct-4 marker (figure 6). Crucial for cardiac lineage formation, Oct-4+ cells make up almost all of the cells on day 0. Capacity of the mESCs to contribute to the pool of CPCs doesn't seem to limit the yield. Instead, cytokines activin A and BMP4 needed titration for optimal amounts each time a new batch was used for differentiation. The best results were obtained with approximately

20% of cells becoming Myl2-eGFP+ in neutral controls at day 9, quantified by flow cytometry.

Directed differentiation protocol promotes commitment to cardiac fate producing a heterogenous, CPC enriched population to plate at day 5. While CPCs were not extensively characterized, their identity as progenitors is evident. The CPCs are capable of differentiating into CMs, but likely also other cells of cardiac lineage (figure 7). Expression of cardiac markers Nkx2.5, Tbx5, Isl1 and Mef2c by day 6 indicates commitment to cardiac lineage (Kattman et al. 2011).

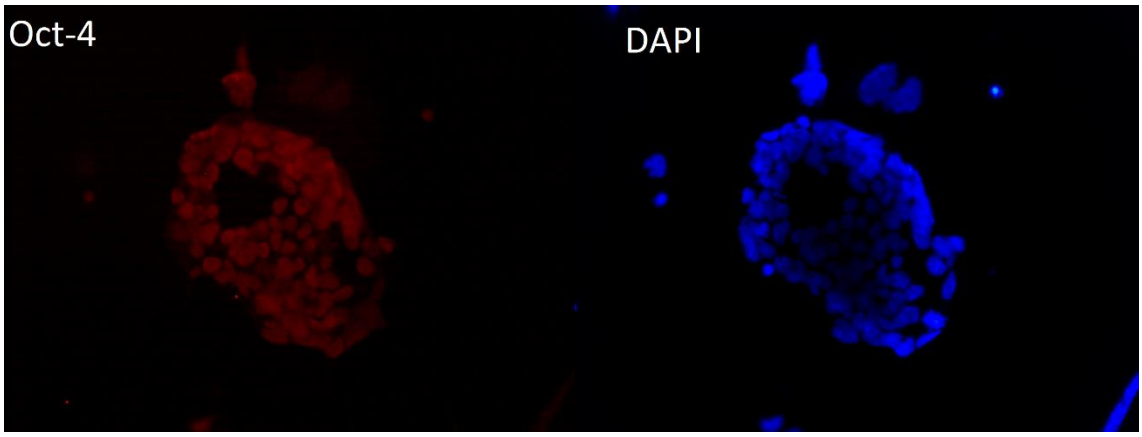

Figure 6. mESC colony of the reporter line stained positive for Oct-4. Oct-4 is a pluripotency marker and DAPI a non-specific staining for nuclei.

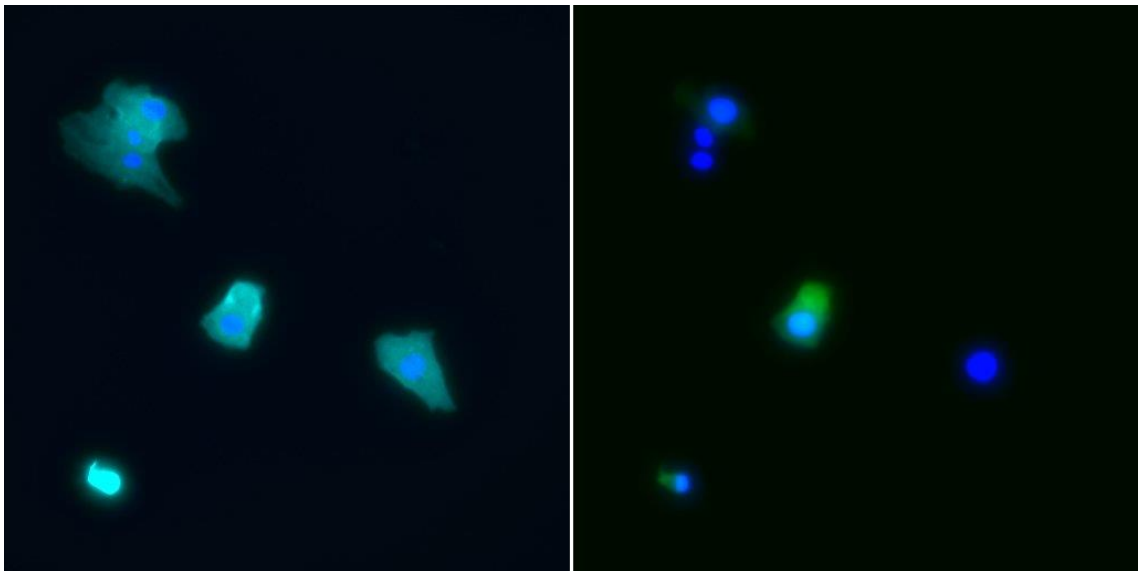

Figure 7. Day 20 double reporter line cells stained for α-smooth muscle actin (α-SMA; cyan) with nuclei stain (blue). α-SMA coincides with Myl2-eGFP in a portion of the cells, while α-SMA also marks some $Myl2^-$ cells that represent non-cardiomyocytes. These $\alpha\text{-SMA}^+$ are not CMs of either ventricular nor atrial phenotype, evident by their lack of endogenous markers Myl2-eGFP and Smyhc3-RFP(Wang et al. 1996). Temporal expression of α-SMA is typical of early stage differentiating CMs (Kern et al. 2014).

6.3 CPCs give rise to CMs in a process susceptible for external regulation

Differentiation and growth of the progenitors is determined by their conditions. The expression of Myl2 on day 9 within cell population is decreased to less than half with day 6-8 addition of retinoic acid (figure 8). Conversely, a known regulator of CPC differentiation XAV939 boosts the signal at least 3,5 times. Sb431542 also produces a clear positive effect as expected. It´s a potent inhibitor of TGF-β pathway and previously reported to enhance differentiation of CPCs. Furthermore, these effects are specific to developmental stage of the cells. Profoundly different effects were found for many of the compounds between d6-8 and d7-9 exposure (figure 8). This difference in response supports the perception that at least majority of the CPCs reach CM stage around late day 7, converting their reaction to stimuli. Interestingly, the effect of retinoic acid gets reversed whereas most of the other library compounds cause a similar response between d6-8 and d7-9.

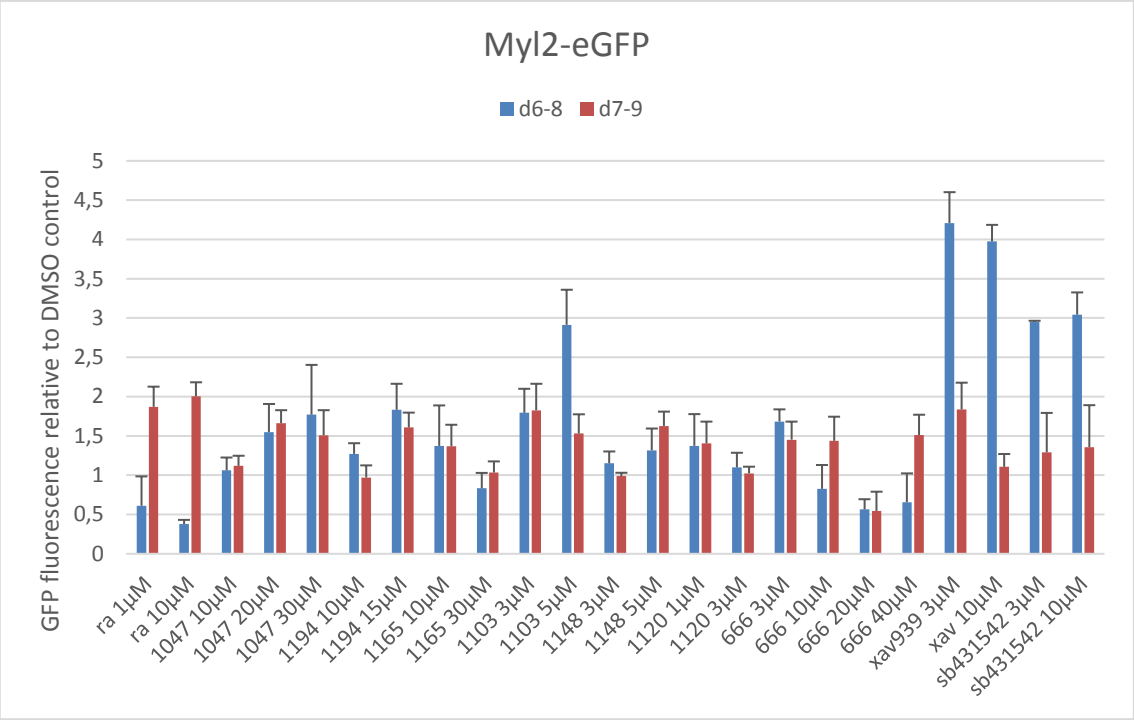

Figure 8. Compound assay results. eGFP fluorescence intensity (mean ± SEM, n=3) relative to DMSO control. 0.1% DMSO was used as a vehicle. Cells were treated with the molecules day 6-8 (in blue) and day 7-9. The effects were quantified with plate reader on day 9 (Pherastar FS 485/520nm). The amount of Myl2-eGFP reporter indicates quantity and maturity of ventricular CMs. Cells' developmental stage defines the effect of different stimuli: Wnt pathway inhibitor XAV939 boosts CPCs' CM yield many fold at earlier stage and not a day later.

One of the library's research molecules was identified to more than double the yield of Myl2-eGFP (figure 8). Only 2 concentrations were tested during this testing phase but the compound also seems to produce a dose dependent effect (figure 9). XAV939 was chosen for strong maximum effect which it induces with both tested concentrations. Other compounds with a less potent effect would only be considered hits with a cutoff activity of 1,5 that of control.

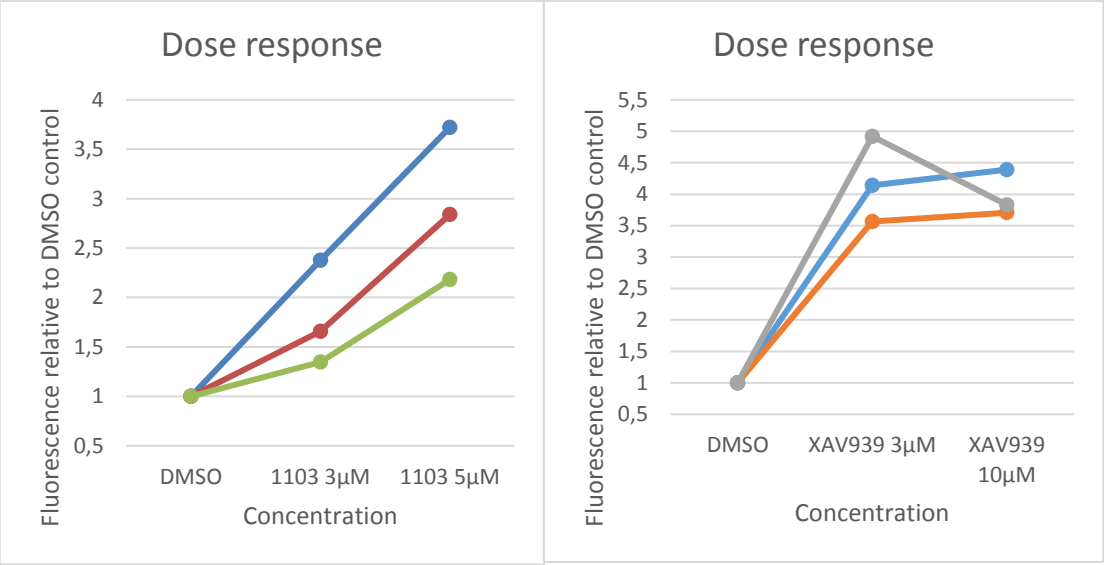

Figure 9. XAV939 and compound 1103 seemed to produce a positive dose response with a clear effect in the screening assay when introduced day 6-8. Results represent three separate assays with four technical replicates averaged.

6.4 Assay quality

Z'-factor is a statistical parameter used to express assay quality and applicability to HTS (Hughes et al. 2011). Day 6-8 assays of myl2-eGFP yielded Z'-factor values 0.88, 0.74 and 0.65 with 10µM XAV939 as maximum signal and DMSO as minimum (Iversen et al. 2012). Values of Z'>0,5 represent excellent performance for screening with significant separation between signals of positive and neutral controls (Iversen et al. 2006; Iversen et al. 2012). The results were reproducible across the three plates, but only the most potent compounds could be clearly identified as hits. The method of producing the CPCs was found unreliable and large number of experiments failed due to low yield of CPCs. These failed plates are easily identified but drive up the cost of the assay.

7. Discussion and conclusion

The goal of this project was to establish a method to test a number of candidate molecules efficiently in a microplate format. The screening assay was to identify

compounds, which might enhance proliferation or differentiation of cardiac progenitors. This small scale screening successfully identified several chemicals enhancing ventricular CM output of CPCs. Developmental stage dependency was simultaneously demonstrated for regulators of common pathways as expected. Proliferative potential declines but is not completely ceased within this early time period of testing.

Various screening assays to a similar end have been published, most based on imaging for quantification. Evidently, the choice between plate reader and imaging based techniques is a compromise. Plate readers are applicable and widely used for high-throughput screening (HTS) assays. HTS utilizing modern plate readers is made simple and quick. Most phenotypic screens are high content screen (HCS) with generally lower throughput. Image based quantification has demonstrated accuracy superior to plate reader in this application in one design (Uosaki and Yamashita 2011).

One compound of certain interest was identified by the assay. Further experimentation will need to be performed to characterize hits like this. Any hits should be verified in secondary assays. Using flow cytometry to determine the number of cells would provide interesting data as to whether more CMs are present and if their Myl2 content differs from the controls.

Reporter line used in the study allows quantification in several time points during test period without having to destroy samples. The results may be further ensured with other methods using the intact samples. The use of reporter line minimizes the number of steps needed to prepare the samples for quantification. This simplifies the process.

The main problem with the developed assay is to reliably produce progenitor cells with appropriate potency. In the process of induction the progenitors lose their potency to develop into CMs. The induction process is affected by such a large variety of factors that they become essentially impossible to completely prepare for. It seems probable that the major cause of defective induction was variability in cell culture materials. Efficient and reproducible production of CPCs is a common challenge among different research groups that have tried to implement medium or large scale assays similar to

this one (Willems et al. 2011). Cryopreserving the CPCs for later use is one way to address the problem. If a large number of CPCs were created at once, properties of this population could be verified before their use in successive screening experiments.

8. References

Ahuja P, Sadek P, Maclellan WR: Cardiac Myocyte Cell Cycle Control in Development, Disease and Regeneration. Physiol Rev 87: 521-544, 2007

Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabe-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisen J: Evidence for cardiomyocyte renewal in humans. Science 324: 98-102, 2009

Bersell K, Arab S, Haring B, Kühn B: Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. Cell 138: 257-270, 2009

Broughton KM, Sussman MA: Empowering Adult Stem Cells for Myocardial Regeneration V2.0. Success in Small Steps. Circ Res. 118: 867-880, 2016

Bruneau BG: Signaling and Transcriptional Networks in Heart Development and Regeneration. Cold Spring Harb Perspect Biol 5: 2013

Buckingham M, Meilhac S, Zaffran S: Building the mammalian heart from two sources of myocardial cells. Nat Rev Genet. 6: 826-35, 2005

Calderon D, Bardot E, Dubois N: Probing Early Heart Development to Instruct Stem Cell Differentiation Strategies. Dev Dyn. 245: 1130-1144, 2016

Cesselli D, Aleksova A, Mazzega E, Caragnano A, Beltrami AP: Cardiac stem cell aging and heart failure. Pharmacol Res. 2017

del Alamo JC, Lemons D, Serrano R, Savchenko A, Cerignoli F, Bodmer R, Mercola M: High throughput physiological screening of iPSC-derived cardiomyocytes for drug development. BBA - Molecular Cell Research, 2016

Drowley L, Koonce C, Peel S, Jonebring A, Plowright AT, Kattman SJ, Andersson H, Anson B, Swanson BJ, Wang Q-D, Brolen G: Human iPSC-Derived Cardiac Progenitor Cells in Phenotypic Screening: A Transforming Growth Factor-b Type 1 Receptor Kinase Inhibitor Induces Efficient Cardiac Differentiation. Stem cell transl med 5: 164-174, 2016

Ellison G, Vicinanza C, Smith A: Adult c-kitpos Cardiac Stem Cells Are Necessary and Sufficient for Functional Cardiac Regeneration and Repair. Cell 154: 827-842, 2013

Eulalio, M. Mano, M. Dal Ferro, L. Zentilin, G. Sinagra, S. Zacchigna, M. Giacca, Functional screening identifies miRNAs inducing cardiac regeneration. Nature 492, 376–381, 2012

Hartman ME, Librande JR, Medvedev IO, Ahmad RN, Moussavi-Harami F, Gupta PP, Chien WM, Chin MT: An Optimized and Simplified System of Mouse Embryonic Stem Cell Cardiac Differentiation for the Assessment of Differentiation Modifiers. PLoS ONE 9: 2014

Hartogh SCD, Passier R: Concise Review: Fluorescent Reporters in Human Pluripotent Stem Cells: Contributions to Cardiac Differentiation and Their Applications in Cardiac Disease and Toxicity. Stem Cells 34: 13–26, 2016

Hastings CL, Roche ET, Ruiz-Hernandez E, Schenke-Layland K, Walsh CJ, Duffy GP: Drug and cell delivery for cardiac regeneration. Adv Drug Deliv Rev 84: 85-106, 2015

Hatzistergos KE, Takeuchi LM, Saur D, Seidler B, Dymecki SM, Mai JJ, White IA, Balkan W, Kanashiro-Takeuchi RM, Schally AV, Hare MJ: cKit+ cardiac progenitors of neural crest origin. PNAS 112: 13051–13056, 2015

Hesse M, Raulf A, Pilz GA, Haberlandt C, Klein AM, Jabs R, Zaehres H, Fu¨gemann CJ, Zimmermann K, Trebicka J, Welz A, Pfeifer A, Röll W, Kotlikoff MI, Steinhäuser C, Götz M, Schöler HR, Fleischmann BK: Direct visualization of cell division using high-resolution imaging of M-phase of the cell cycle. Nat Commun 3: 1076, 2012

Hosoda T, D'Amario D, Cabral-Da-Silva MC, Zheng H, Padin-Iruegas ME, Ogorek B, Ferreira-Martins J, Yasuzawa-Amano S, Amano K, Ide-Iwata N, Cheng W, Rota M, Urbanek K, Kajstura J, Anversa P, Leri A: Clonality of mouse and human cardiomyogenesis in vivo. Proc Natl Acad Sci USA 106: 17169-17174, 2009

Hsieh PCH, Segers VFM, Davis ME, MacGillivray C, Gannon J, Molkentin JD, Robbins J, Lee RT: Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. Nat Med 13: 970–974, 2007

Hughes J, Rees S, Kalindjian S, Philpott K: Principles of early drug discovery. Br J Pharmacol 162: 1239-1249, 2011

Iversen PW, Eastwood BJ, Sittampalam GS, Cox KL: A Comparison of Assay Performance Measures in Screening Assays: Signal Window, Z′ Factor, and Assay Variability Ratio. Journal of Biomolecular Screening 11: 247-252, 2006

Iversen PW, Beck B, Chen YF, et al: HTS Assay Validation. Available from: https://www.ncbi.nlm.nih.gov/books/NBK83783/. In: Sittampalam GS, Coussens NP, Brimacombe K, et al.: Assay Guidance Manual. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences, 2012

Jaroszeski MJ, Radcliff G: Fundamentals of flow cytometry. Mol Biotechnol 11: 37-53, 1999

Jazwinska A, Sallin P: Regeneration versus scarring in vertebrate appendages and heart. J Pathol 238: 233-246, 2015

Kajstura J, Urbanek K, Perl S, Hosoda T, Zheng H, Ogórek B, Ferreira-Martins J, Goichberg P, Rondon-Clavo C, Sanada F, D'Amario D, Rota M, Del Monte F, Orlic D, Tisdale J, Leri A, Anversa P: Cardiomyogenesis in the adult human heart. Circ Res 107: 305-315, 2010

Karathanasis SK: Regenerative Medicine: Transforming the Drug Discovery and Development Paradigm. Cold Spring Harb Perspect Med 4: 2014

Kattman SJ, Witty AD, Gagliardi M, Dubois NC, Niapour M, Hotta A, Ellis J, Keller G: Stage-Specific Optimization of Activin/Nodal and BMP Signaling Promotes Cardiac Differentiation of Mouse and Human Pluripotent Stem Cell Lines. Cell stem cell 8, 228-240, 2011

Kern S, Feng HZ, Wei H, Cala S, Jin JP: Up-regulation of alpha-smooth muscle actin in cardiomyocytes from non-hypertrophic and non-failing transgenic mouse hearts expressing N-terminal truncated cardiac troponin I. FEBS Open Bio. 4: 11-17, 2014

Kikuchi K, Poss KD: Cardiac regenerative capacity and mechanisms. Annu Rev Cell Dev Biol 28, 719–741, 2012

Kikuchi K, Holdway JE, Werdich AA, Anderson RM, Fang Y, Egnaczyk GF, Evans T, Macrae CA, Stainier DY, Poss KD: Primary contribution to zebrafish heart regeneration by gata4+ cardiomyocytes. Nature 464: 601-605, 2010

Kokkinopoulos I, Ishida H, Saba R, Coppen S, Suzuki K, Yashiro K: Cardiomyocyte Differentiation From Mouse Embryonic Stem Cells Using a Simple and Defined Protocol. DEVELOPMENTAL DYNAMICS 245: 157–165, 2016

Koudstaal S, Jansen of Lorkeers SJ, Gaetani R, Gho JMIH, van Slochteren FJ, Sluuter JPG, Doevendans PA, Ellison GM, Chamuleau SAJ: Concise Review: Heart Regeneration and the Role of Cardiac Stem Cells. Stem Cells Transl Med 2: 434-443, 2013

Kurosawa H: Methods for inducing embryoid body formation: In vitro differentiation system of embryonic stem cells. 2007

Laflamme MA, Murry CE: Heart regeneration. Nature 473: 326-335, 2011

Längle D, Halver J, Rathmer B, Willems E, Schade D: Small molecules targeting in vivo tissue regeneration. ASC Chem Biol 9: 57-71, 2014

Le TYL, Chong JJH: Cardiac progenitor cells for heart repair. Cell Death Discovery 2: 16052, 2016

Lenz H-J, Kahn M: Safely targeting cancer stem cells via selective catenin coactivator antagonism. Cancer Sci 105: 1087-1092, 2014

Liu W, Foley AC: Signaling pathways in early cardiac development. Syst Biol Med 3: 191-205, 2011

Malliaras K, Zhang Y, Seinfeld J, Galang G, Tseliou E, Cheng K, Sun B, Aminzadeh M, Marban E: Cardiomyocyte proliferation and progenitor cell recruitment underlie therapeutic regeneration after myocardial infarction in the adult mouse heart. EMBO Mol Med 5: 191–209, 2013

Mollova M, Bersell K, Walsh S, Savla J, Das LT, Park SY, Silberstein LE, Dos Remedios CG, Graham D, Colan S, Kühn B: Cardiomyocyte proliferation contributes to heart growth in young humans. Proc. Natl. Acad. Sci. U.S.A. 110: 1446–1451, 2013

Noseda M, Peterkin T, Simoes FC, Patient R, Schneider MD: Cardiopoietic factors: extracellular signals for cardiac lineage commitment. Circ Res 108: 129-152, 2011

Paige SL, Plonowska K, Xu A, Wu SM: Molecular regulation of cardiomyocyte differentiation. Circ res 116: 341-353, 2015

Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN, Sadek HA: Transient regenerative potential of the neonatal mouse heart. Science: 331: 1078-80, 2011

Russell JL, Goetsch SC, Aguilar HR, Frantz DE, Schneider JW: Targeting Native Adult Heart Progenitors with Cardiogenic Small Molecules. ACS Chem Bio. 7: 1067-1076, 2012

Sahara M, Santoro F, Chien KR: Programming and reprogramming a human heart cell. EMBO J 34: 710-738, 2015

Santini MP, Forte E, Harvey RP, Kovacic JC: Developmental origin and lineage plasticity of endogenous cardiac stem cells. Development 143: 1242-1258, 2016

Schade D, Plowright AT: Medicinal Chemistry Approaches to Heart Regeneration. J Med Chem 58: 9451-9479, 2015

Senyo SE, Steinhauser ML, Pizzimenti CL, Yang VK, Cai L, Wang M, Wu TD, Kern JLG, Lechene CP, Lee RT: Mammalian heart renewal by pre-existing cardiomyocytes. Nature 493: 433-436, 2013

Spiering S, Davidovics H, Bushway PJ, Mercola M, Willems E: High Content Screening for Modulators of Cardiac Differentiation in Human Pluripotent Stem Cells. Methods Mol Biol; 1263: 43–61, 2015

Swinney DC, Anthony J: How were new medicines discovered? Nat. Rev. Drug Discov. 10: 507–519, 2011

Szema AM, Dang S, Li JC. Emerging Novel Therapies for Heart Failure. Clin Med Insights: Cardiology 9: 57-64, 2015

Takahashi T, Lord B, Schulze PC, Fryer RM, Sarang SS, Gullans SR, Lee RT: Ascorbic Acid Enhances Differentiation of Embryonic Stem Cells Into Cardiac Myocytes. Circulation 107: 1912-1916, 2003

Takeuchi T: Regulation of cardiomyocyte proliferation during development and regeneration. Develop. Growth Differ 56: 402-409, 2014

Tam PPL, Parameswaran M, Kinder SJ, Weinberger RP: The allocation of epiblast cells to the embryonic heart and other mesodermal lineages: the role of ingression and tissue movement during gastrulation. Development 124: 1631–1642, 1997

Uosaki H, Magadum A, Seo K, Fukushima H, Takeuchi A, Nakagawa Y, Moyes KW, Narazaki G, Kuwahara K, Laflamme M, Matsuoka S, Nakatsuji N, Nakao K, Kwon C, Kass DA, Engel FB, Yamashita JK: Identification of Chemicals Inducing Cardiomyocyte Proliferation in Developmental Stage-Specific Manner with Pluripotent Stem Cells. Circ Cardiovasc Genet 6: 624–633, 2013

Uosaki H, Yamashita JK: Chemicals Regulating Cardiomyocyte Differentiation, Embryonic Stem Cells: The Hormonal Regulation of Pluripotency and Embryogenesis, InTech, 2011

Uygur A, Lee RT: Mechanisms of cardiac regeneration. Dev Cell 36: 362-374, 2016

Valiente-Alandi I, Albo-Castellanos C, Herrero D, Sanchez I, Bernad A: Bmi1+ cardiac progenitor cells contribute to myocardial repair following acute injury. Stem Cell Res Ther 7: 100, 2016

van Berlo JH, Kanisicak O, Maillet M, Vagnozzi RJ, Karch J, Lin SCJ, Middleton RC, Marban E, Molkentin JD: c-kit+ cells minimally contribute cardiomyocytes to the heart. Nature 509: 337–341, 2014

van Vliet P, Wu SM, Zaffran S, Pucéat M: Early cardiac development: a view from stem cells to embryos. Cardiovasc Res 96: 352-362, 2012

Vincent SD, Buckingham ME: 2010 Vincent SD, Buckingham ME: How to make a heart: The origin and regulation of cardiac progenitor cells. Curr. Top. Dev. Biol. 90: 1–41, 2010

Wang GF, Nikovits W Jr, Schleinitz M, Stockdale FE: Atrial chamber-specific expression of the slow myosin heavy chain 3 gene in the embryonic heart. J Biol Chem 271: 19836–19845, 1996

Willems E, Lanier M, Forte E, Lo F, Cashman J, Mercola M: A Chemical Biology Approach to Myocardial Regeneration. J Cardiovasc Transl Res. 4: 340–350, 2011

Xin M, Olson EN, Bassel-Duby R: Mending broken hearts: cardiac development as a basis for adult heart regeneration and repair. Nat Rev Mol Cell Biol 14: 529-541, 2013

Zelarayán LC, Zafiriou MP, Zimmermann W: Myocardial Pharmacoregeneration. Regenerative Medicine-from Protocol to Patient 5: p 111-143, third edition, Springer, 2016